Some Observations upon Spirostomum ambiguum (Ehrenberg).

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With Plates 22 and 23 and 9 Text-figures.

Contents.

1. Historical.

The genus Spirostomum was first mentioned by Ehrenberg, but no definition nor description was given. Its systematic position and the question of the number and identity of the species contained in it was a subject for discussion for many years. Later, Dujardin (7) gave a very satisfactory description of the genus in the following words :

'Corps cylindrique très-allongé et très-flexible, souvent tordu sur lui-même, couvert de cils disposés suivant les stries obliques ou en hélice de la surface ; avec une bouches située latéralement au delà du milieu, à l'extrémité d'une rangée de cils plus forts.'

He recognized, however, only S p i r o s t o m u m a m b i g u u m as a true species.

It is to Dr. Stein (28) that we are indebted for a comprehensive and beautifully illustrated description of the genus, together with a detailed account of the vicissitudes of nomenclature through which it had passed since its discovery by Ehrenberg. Stein recognizes two species of Spirostomum, S . a m b i g u u m (Ehrenberg) and S . t e r e s (Claparède et Lachmann) (5). Previously Perty (24) had included another form in the genus, and to it he gave the name S . s e m i - v i r e s c e n s . His observations, which were founded on a single specimen, are regarded by Stein as being of too superficial a nature to justify the creation of a new species. He believed it to be merely a variety of S p i r o s t o m u m a m b i g u u m .

Pénard (23) recognizes S . a m b i g u u m and S . t e r e s as true species, and to these adds S . f i l u m . This latter, although described by Ehrenberg as U r o l e p t u s f i l u m , was placed tentatively in the genus Spirostomum by Bütschli (3) and Claparède et Lachmann. Stein does not classify it among his species. None of the above workers, with the exception of Ehrenberg, had seen it personally, but has relied upon Ehrenberg's figures for their data. Pénard has actually seen it, and feels quite certain that it justifies the position he has given it as a third species of the genus Spirostomum.

2. Material and Methods.

The Spirostoma from which the cultures were started were obtained from ponds in North Cheshire, principally in the neighbourhood of Ringway and Styal near Manchester. They were most numerous in rather deep ponds, with muddy bottoms covered with decaying vegetable matter, and with Lemna covering the surface. A good supply was obtained during the drought of the summer of 1921 whilst the ponds were low and the water fairly concentrated, but all through the autumn and winter, though a large number of ponds were visited, including those visited in the summer, very few specimens were obtained. In May and June they became plentiful again and obviously were multiplying rapidly, since many dividing forms were collected.

Fixation.—The fixation of Spirostomum ambiguum is very unsatisfactory because the animal possesses very highly developed powers of contraction. Attempts to narcotize them with chloroform, ether, carbon-dioxide, or by the action of Epsom salts proved unsuccessful, though the narcotics were used in minute quantities and in very dilute solutions.

Bouin's solution and hot or cold Schaudinn's solution are both good fixatives for the nuclear structures. For whole mounts hot Schaudinn's solution gives the best results. since the contraction of the cell is less with this fixative. When whole mounts were required, the animals, together with as small a quantity of culture fluid as was possible, were placed on a slide smeared with egg albumen. The hot Schaudinn's solution was dropped rapidly on the animals whilst they were extended. Contraction of the whole body invariably took place, but by this method there was no shrinkage of the endoplasm from the ectoplasm. This contraction of the body was not really very disastrous, since the main outline of the meganucleus and its relative position were easily studied in the living animal and fixed preparations were required for detailed study.

It was found best, whenever possible, to starve the material

for a few hours prior to fixation. This had the effect of removing all the undigested food which otherwise would have obscured the details of nuclear structure.

Animals destined to be sectioned were fixed in bulk in a watch-glass with warm Schaudinn's solution or with Bouin's solution. The fixative was washed away and the animals were removed to a narrow tube where they were treated with the different percentages of alcohol. When in 50 per cent. alcohol they were lightly tinged with borax carmine to facilitate their orientation in the paraffin wax. They were cleared with xylol. After they had been cleared they were transferred to a watch-glass containing xylol in which paraffin wax was gradually dissolved. By means of a warm, fine pipette they were transferred to pure wax, contained in a clean porcelain dish, and left in the embedding oven for about two minutes. The animals, together with some of the wax, were dropped upon a glass slide which previously had been smeared with egg albumen. They were orientated with a warm needle before the wax solidified. The solidified wax was then shaved into small blocks.

Staining.—Borax carmine, alum carmine, paracarmine, Delafield's haematoxylin, aqueous iron haematoxylin, and Dobell's alcoholic iron haematoxylin all proved to be useful stains. Aqueous iron haematoxylin gave the best results for sections, but the alcoholic modification of the stain was generally used for whole mounts, since in aqueous solution the animals often became detached from the slide.

Methyl green in 1 per cent. acetic was used for fixing and staining animals not needed as permanent preparations.

The method generally used for clearing whole mounts was to soak the dehydrated preparations in clove oil for about twenty minutes and to wash away the oil with xylol before mounting in Canada balsam. Hairs were used to support the coverslips because there is a tendency for the unsupported coverslips to crush so large an animal as Spirostomum.

Observations on Living Specimens.—For isolation of an animal for repeated observations it was found best

to use a hollowed slide, which, when it was not under observation, was kept in a moist chamber. Although hanging drops were used at first, they were abandoned later, when it was found that when the animal moved to the edge of the drop, which it normally did, it rapidly disintegrated there.

To facilitate observations on living Spirostoma, Caragheen extract was used. This slows down their movements considerably ; but, since their shape becomes somewhat distorted with the density of the medium and disintegration often follows, it is not advisable to use it when keeping the animals under observation for a long period. It was very useful, however, for the study of ciliary structures.

Feeding Methods.—For following the course of ingested material, finely powdered carmine or Indian ink in culture solution were both used. A dilute solution of milk in culture solution was also used, as was also finely powdered yolk of egg.

3. Observations on the Morphology of Spirostomum ambiguum.

As a description of the general morphology and movements of Spirostomum ambiguum Dr. Stein's (28) excellent account has not been improved upon. It will be sufficient here, before passing on to a detailed account of the various structures, to mention that Spirostomum ambiguum is a large, elongated ciliate belonging to the order Heterotricha. The peristomial groove, which terminates in the mouth, is lateral in position, but the distance of the mouth from the anterior end of the body may vary considerably in different individuals. The peristomial membranellae extend from the extreme anterior end to the mouth, around which they curve in a spiral manner. The meganucleus is long and moniliform. The numerous small micronuclei are situated close to the meganucleus.

Contractile Vacuole.—Spirostomum ambiguum is bounded externally by a relatively thin layer of ectoplasm, on the outer side of which is the thin cuticle. The ectoplasm

has none of the coarsely vacuolated structure characteristic of the endoplasm.

The contractile vacuole lies at the posterior end of the animal. There is a long feeding canal stretching from the anterior end of the animal to the contractile vacuole into which it opens. When distended with fluid the contractile vacuole fills almost completely the posterior end of the animal, and only a very narrow band of endoplasm lies along one side of it, down which the food passes to the median cytopyge (see p. 408). The relative size of the vacuole to that of the whole body, and also its shape, varies with the variety of Spiro-stomum ambiguum, and this point will be dealt with later.

Transverse sections of Spirostomum ambiguum show that the outline of the contractile vacuole and its canal is perfectly definite. The vacuole and its canal lie immediately below the ectoplasm (see Text-figs. 1 and 2). When full the canal protrudes far into the endoplasm, by which it is almost completely surrounded.

When contraction of the vacuole is about to take place the liquid passes down the feeding canal, which normally closes behind it, and into the vacuole proper, which becomes very much distended. Normally the voiding of the contents to the exterior immediately follows this, but in some cases, particularly in partially narcotized animals, and also often in animals kept for a long time in hollow slides with a small quantity of liquid, evacuation does not immediately take place. The animal continues to swim about with a large, closed vacuole at the posterior end. When the vacuole is about to be emptied an opening is formed at the posterior end at the base of a slight depression ; the body-walls surrounding the vacuole contract from before backwards, the liquid is forced out of the opening and the vacuole disappears. This complete contraction of the contractile vacuole gives the posterior end of the animal a compressed appearance. The new vacuole and feeding canal are formed in exactly the same position as was occupied by the preceding one.

Endoplasm and Nuclei.—The endoplasm consists of large vacuoles separated by narrow meshes of fairly fluid protoplasm. In the endoplasm lies the long moniliform mega-nucleus. In the living animal its form can be followed quite easily, since its denser structure and greater powers of refraction readily distinguish it from the rest of the protoplasm. Normally it consists of a single unbranched chain, extending in a fairly straight or slightly zigzag manner from the anterior end to the contractile vacuole. The lobes, which vary con-

TEXT-FIGS. 1 AND 2.
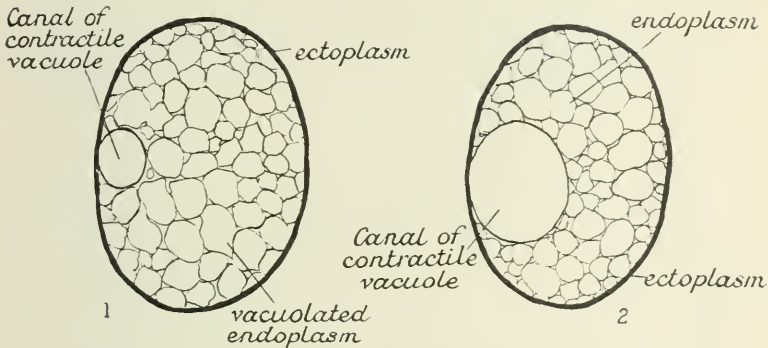
siderably in size, are joined together by commissures which may be either almost as wide as the lobes themselves or very narrow. The lobes vary also in number. The least number I have ever seen in any member of the large variety was ten and the greatest number was fifty.

In some animals, the nucleus, although it was normal in length, had an unconstricted, vermiform shape and in places was slightly coiled. In all other respects the individuals seemed quite normal and the position of the mouth (see part on Fission) did not point to any very recent or immediately approaching fission. I found, however, by isolating these individuals and keeping them under observation for a number of hours which varied with the individual, that lobation eventually did take place and that it was in this case delayed for a much longer

period after fission than usual. A similar phenomenon was observed by Johnson (**13**) during his study of the Stentors.

Stein (**28**) describes cases where the meganucleus was only a quarter of the body length, was not lobated, and lay in the anterior end of the body. These were, I feel certain, also stages in fission.

The meganucleus is surrounded by a nuclear membrane which adheres firmly to the nucleoplasm. It is best shown in individuals which have been fixed and stained in methyl green and acetic, especially if the cytoplasm has been teased out prior to fixation.

In preparations well fixed and stained with iron haematoxylin the internal structure of the meganucleus is plainly visible. It consists of numerous granules, which stain deeply with iron haematoxylin, embedded in a fairly homogeneous matrix.

Greenwood (**9**) terms these deeply staining granules macrosomes, and describes in addition to these other minute granules which do not stain deeply with haematoxylin but do so with borax carmine. These latter she calls microsomes. In my preparations with borax carmine the nucleus seems to have a finely granular appearance, but the large granules (i. e. Greenwood's macrosomes) do not combine with this stain.

In preparations stained with iron haematoxylin the macrosomes are seen to be present in both the commissures and lobes of the nucleus ; but if the commissures are very narrow they are confined to the lobes alone. The macrosomes vary in size from minute dots barely visible at a high magnification to masses up to 10 μ or more in breadth (Pl. 22, figs. 1, 2, and 3). Often these granules are surrounded by lightly staining areas. Since these are not always present it is possible that they are due to the fixative and are not to be interpreted as part of the normal nuclear structure. The macrosomes vary greatly in shape ; they are generally round, but may be oval, pear-shaped, or even roughly oblong. The medium-sized granules often show a single vacuole in the centre (Pl. 22, fig. 1, *vac.*), whilst invariably within the large masses one or more vacuoles

are present (Pl. 22, fig. 2, *vac.*). Occasionally as many as five vacuoles have been seen in one large macrosome, each vacuole being separated from those adjacent to it by strands of the darkly staining substance of which the macrosome is composed.

The presence of small non-vacuolated granules, and medium or large-sized ones containing one or more vacuoles, in the same lobe of the nucleus is quite common. Sometimes all the granules present in the nucleus are without vacuoles, whilst in others they are all large with many vacuoles.

Collin (6) describes similar macrosomes and microsomes in the nuclei of Acinetaria. The microsomes he believes to be true chromatin grains, whilst the macrosomes he regards as true nucleoli.

Owing to the fact that I have not had time or opportunity as yet to study these structures in detail in Spirostomum, I do not propose to offer any speculation as to their nature. I should, however, like to add that since there exist all degrees of vacuolation and non-vacuolation, and since whenever large masses with numerous vacuoles are present the actual number of masses is small, it seems to me very probable that the large vacuolated masses (i. e. Greenwood's macrosomes) are formed by a flowing together of a number of the smaller macrosomes and a subsequent vacuolation from several centres.

Animals having large multivacuolated macrosomes in their meganuclei do not seem to be otherwise abnormal, and show no signs of degeneration in the cytoplasmic structures. Since the degree of vacuolation seems to be independent also of the degree of growth after fission it seems quite probable, as Greenwood suggests, that it is due to diet or to some temporary condition of the culture medium.

The Micronuclei.—The micronuclei of S p i r o s t o m u m a m b i g u u m are minute in size and difficult to find. They completely escaped the notice of Stein (28). Maupas (17) was the first to discover their existence. They lie close to, but are not attached to, the meganucleus (Pl. 22. fig. 1. *M.N.*). In structure they consist of a central endosome, presumably

composed of chromatin, since it stains darkly with the various haematoxylin and carmine stains and with methyl green. This endosome seems to be homogeneous and is surrounded by a pale area or halo, around which there appears to be a definite membrane.

In his summary of our knowledge of the multinucleate ciliates Calkins (**4**) says that ' Balbiani, in his earlier work at least, held that the number of micronuclei is always the same as the macronuclei, or in beaded forms, as many as there are segments of the macronucleus '. He goes on to say that Maupas (**18**), Gruber, Bütschli, and others disproved this view. They found that the numbers were the same in some ; in other cases, of which Stentor is an example, the micronuclei outnumber the segments of the macronucleus ; whilst in other forms, including S p i r o s t o m u m a m b i g u u m, the segments of the macronucleus outnumber the micronuclei. I can fully endorse the statement that the micronuclei do not correspond in number to the lobes of the meganucleus, for I have seen individuals in which a number of lobes had no micronuclei near to them, whilst others have two, or in some cases three, four, or five to each lobe. The micronuclei are found opposite to the commissures as well as opposite to the lobes.

In the majority of individuals which I examined, however, the number of lobes of the meganucleus is greater than the number of micronuclei. In a number of cases there have been nearly twice as many lobes of meganucleus as micronuclei present. On the other hand, quite an appreciable number of individuals have been observed in which the micronuclei were approximately equal in number to the lobes of the meganucleus or slightly exceeded them. In one case where the number of lobes in the meganucleus was only ten, twenty-six micronuclei were present.

From these observations it seems clear that, subsequent to fission, in the change from a vermiform to moniliform type of nucleus, there is no correlation between the number of constrictions appearing in the meganucleus and the number of micronuclei present in the daughter Spirostomum.

Abnormalities in the Form of the Meganucleus.
—A number of individuals from different cultures have been
found in which the meganucleus was abnormal. These observa-
tions include individuals of both the major and minor varieties
(see below).

One member of the major variety was found whose mega-
nucleus had a short branch, consisting of two lobes and a
commissure, given off from one of the commissures. This was
the only case of a branched meganucleus met with.

A fairly common abnormality was the division of the mega-
nucleus into two pieces ; in one case three pieces of mega-
nucleus were present. Such conditions might be brought
about by the snapping of a delicate commissure. Another
method was revealed, however, whilst watching a normal
individual divide. During the contraction of the meganucleus
towards the anterior end of the animal, the posterior end of
the meganucleus was seen to come apart from the rest and to
follow as a separate small oval fragment in the wake of the
rest. Elongation subsequently took place, and, when the
constriction of the cell occurred, the anterior daughter con-
tained a whole daughter meganucleus, whilst the posterior
daughter contained its share of the major fragment and the
separated minor fragment. Each of these latter gave rise to
a piece of moniliform meganucleus.

The most interesting cases of abnormalities, however, were
found in a five months' old culture of the minor variety of
Spirostomum ambiguum. The cilia of all were normal,
but the protoplasm seemed denser and more granular than
usual, although it showed no signs of the vacuolation usually
associated with degenerate forms. The meganucleus had lost
its moniliform appearance and lay collected in masses in the
endoplasm. In some cases it took the form of three or four
rounded masses separated from one another by quite distinct
gaps. In one case the meganucleus was represented by a big
sphere in the anterior end separated by a wide space from the
remainder, which took the form of four lobes of the normal
moniliform type.

In three cases the meganucleus was broken up into three or more rounded spheres lying in the endoplasm, the hindermost of which had passed down the body and lay as a small refractive ball at the extreme posterior end. Stained preparations of two of these animals showed that their meganucleus was composed of darkly staining granules packed more closely together than they normally are but not vacuolated. Each sphere of meganucleus was surrounded by micronuclei. The remaining individual was isolated on a well-slide, supplied with a little of the original culture medium and put into a moist chamber. After about twenty-four hours it was again observed. The spheres of the meganucleus were in practically the same position in the anterior part, but the posterior sphere had disappeared. Whether it had been absorbed or had passed out of the body I cannot say; but its position in relation to the cytopyge on the previous day seemed to suggest that the latter fate had befallen it. The animal was kept two more days without any important changes taking place. At the end of that time it died.

The culture in which these cases were found was an old leaf one. The animals in it were very few and no case of division was observed while it was under observation. From the lack of food vacuoles in the animals it was obvious that the culture was in an impoverished state, and these abnormalities were no doubt due to starvation.

4. Methods of Cultivation.

The first attempts to form cultures of Spirostomum ambiguum were made with hay infusions. A similar solution to Woodruff's standard hay infusion (31) was made, the formula being 10 grammes of chopped hay in 1 litre of tap-water, and raised to the boiling-point for a few minutes. A culture basin containing a quantity of this fluid was inoculated with a few Spirostoma. In a few hours it was found that all the animals had died. Experiments were then made with 75 per cent., 50 per cent., and 25 per cent. dilutions of the fluid with

tap-water. In the stronger solutions the Spirostoma died almost immediately, whilst although those in the weakest solutions lingered for a few days, they showed no signs of multiplying and ultimately locomotion was suspended and disintegration followed.

During his work upon Spirostomum teres, Maupas (19) fed the material upon a solution of flour in water, which he added to their own pond water. In order to find out whether a similar medium would suit Spirostomum ambiguum, 0·150 grm. of flour was added to 100 c.c. of tap-water and boiled for ten minutes, these being the amounts used successfully by Calkins (4) for Uroleptus mobilis. Varying volumes of this solution were added to culture dishes and to test-tubes which contained Spirostoma together with pond-water and a little débris. No marked success followed this experiment. In two cases the test-tube cultures lived for some days but no division was seen. Mixtures of flour, hay, and pond-water in various proportions, and solutions of Lemco and Vitmar were all in turn tried without any success.

Pond-water, together with the slimy, decaying leaves from the bottom of ponds, was boiled for about ten minutes in order to free it from any organisms which might be present. The boiled leaves were placed into test-tubes with about 10 c.c. of the water in which they had been boiled. The tubes were filled up with pond-water boiled to free it from any organisms. At first approximately 2 c.c. of Woodruff's standard hay infusion was added. This hay infusion was cooled and then allowed to stand open to the air for twenty-four hours before it was added to the cultures. By this means the hay infusion was inoculated with a plentiful supply of bacteria. That it is necessary to use newly made infusions is shown by the work of Peters (25), who showed that the maximum development of bacteria in a hay infusion is reached in approximately the first three days. Further, Hargitt and Fray (10) have isolated from old hay infusions many kinds of bacteria which are toxic to Paramoecium. It is not improbable therefore that these old infusions contain bacteria which are toxic to other ciliates as

well. Later, the addition of hay infusion was found to be unnecessary.

The tubes were allowed to stand for not less than four days at a constant temperature of $20°$ C. or for a longer period at a lower temperature in order that bacteria might multiply and decomposition of the leaves and débris set in. Each tube was then inoculated with a few Spirostoma.

In order to discover whether any one particular kind of leaf is more suitable for the cultivation of these animals than another, cultures were made of oak leaves, beech leaves, rushes, and leaves of potomageton respectively. It was found, however, that no individual leaf gave such good results as did a mixture of several different kinds.

At first no multiplication of the Spirostoma took place, although in the majority of the tubes the animals remained alive. After a week had elapsed a smell of decay, in which the odour of sulphuretted hydrogen could be detected easily, issued from the tubes. The cultures darkened in colour, and in a number of cases microscopical investigation revealed the presence of Beggiatoa and numbers of minute green flagellates. The Spirostoma then began to increase rapidly until, about a month after the making of the cultures, they were present in large numbers.

A comparison of these cultures with the ponds in which Spirostomum ambiguum is numerous seems to show that by this method conditions approximately similar to those of their natural environment are obtained artificially. The odour of sulphuretted hydrogen, so noticeable when collecting in the type of pond in which Spirostomum ambiguum is numerous, evidently arises from the decaying vegetable matter. Lauterborn (**14**) believes its presence to be characteristic of the environment necessary to what he calls a 'sapropelic' fauna, and Spirostomum ambiguum figures in his list of such sapropelic organisms.

There seems to be a direct relation between the presence of sulphuretted hydrogen and a thriving condition of the Spirostoma. When the amount of sulphuretted hydrogen is

very great the Spirostoma die. Whether the Spirostoma are directly dependent upon the sulphuretted hydrogen or upon some product of protein decomposition during which sulphuretted hydrogen is liberated, or whether the kind of bacteria upon which they flourish best is dependent upon it, I am at present unable to determine. The last supposition, however, seems the most likely.

While these experiments were being made excellent cultures of A m o e b a p r o t e u s were being obtained by Sister Monica Taylor's wheat method (29). Since large ciliates were often plentiful in such cultures it seemed probable that S p i r o - s t o m u m a m b i g u u m might be grown in a similar medium. To test this assumption, two wheat grains, boiled to stop germination, were put into a test-tube, which was then filled up with aquarium water, previously boiled to free it from any living organisms. The tubes were allowed to stand in the incubator at a temperature of $20°$ C. from four to five days to favour the development of a thick bacterial growth. Spirostoma were then added. Excellent results were obtained from this method, thicker cultures being obtained than from the former cultures. In addition to the Spirostoma, Chilomonas, green flagellates, and pink bacteria often developed in these cultures. The wheat cultures are not only easier to prepare, but have the additional advantage that the bacterial food-supply of the ciliata, and therefore the cultures themselves, lasts longer than it does in the leaf-extract medium.

It is interesting to note that long, narrow test-tubes seem to be necessary for the success of these cultures. All attempts to cultivate Spirostomum in either leaf or wheat extract in shallow, wide dishes were unsuccessful. Spirostomum evidently thrives best in deep water where the surface area, and therefore the amount of dissolved oxygen, is small. That it was not the shallowness of the medium which killed the cultures was shown by attempting to grow Spirostoma in wide, deep jars, when the result, or rather the lack of result, was the same as in the case of the wide, shallow dishes.

5. Food Cycle.

In Spirostomum ambiguum from a healthy culture numbers of large, round food-balls can be observed throughout the endoplasm. These food-balls may be formed of very small green flagellates, clumped closely together to give them a morula-like appearance ; others are brownish in colour and are composed of compact masses of bacteria. Some large, pink-coloured balls are present owing to the animal having fed upon pink bacteria present in the culture. On one or two occasions I have seen Chilomonads in the endoplasm, enclosed in a large fluid vacuole. That these Chilomonads were not yet dead was shown by their undulating movements. It is not usual, however, for Spirostomum ambiguum to ingest anything so large.

In some cultures the food-bodies were entirely bacterial, in others bacteria and flagellates mixed together formed the food-balls, whilst in others the food-balls were composed entirely of green flagellates. The animals were in a flourishing state in all three cases.

Individual Spirostoma were examined for the presence of fluid vacuoles surrounding the food-balls. In the case of bacterial food-balls lying anterior to, or slightly posterior to, the mouth, distinct fluid vacuoles could be seen encircling them. These vacuoles were absent from balls close to the posterior end. Vacuoles similarly encircled balls of bacteria mixed with flagellates. In the case of flagellate balls, only a very thin film of fluid could be detected, or in many cases the balls seemed to be embedded in the coarsely vacuolated endoplasm, without the intervention of a vacuole, whatever their position in the animal might be.

It was soon realized that a definite circulation of the food took place in the endoplasm. An attempt to trace the cycle was made by placing individuals in tap-water for a sufficient length of time to allow all the food to pass out of the body, and then isolating them in well-slides containing some culture solution rich in green flagellates. Unfortunately all the

animals treated in this way refused to feed. After many unsuccessful attempts a few animals were persuaded to feed from such a culture solution in small test-tubes.

The food is wafted down the long peristomial groove to the cytostome by the peristomial membranellae. At the base of the cytostome the food is gathered into a sphere, which varies considerably in size from a ball only just visible under the $\frac{2}{3}''$ objective, to one-half the width of the animal's body. The food-ball then passes forward towards the anterior end of the body. Its movement is, comparatively speaking, rapid. On reaching the anterior end of the body it moves to the posterior end in a course parallel to its former one. After regaining a position approximately level with the cytostome its progress becomes much slower, and with many halts it passes down the side of the contractile vacuole, along the narrow strip of endoplasm found in this region, to the cytopyge. This cytopyge appears at the base of a slight depression situated in the middle of the posterior end. The undigested material is evacuated slowly, one sphere at a time.

Since the movement of the food-balls at the posterior end of the animal is so slow, there is often an accumulation of these spheres in the neighbourhood of the contractile vacuole.

The course followed by flagellate or bacterial food-bodies is shown in Text-fig. 4.

In order to discover whether the course taken by food-balls was similar in the case of substances of no food value, animals were taken straight out of the culture and put directly into a suspension of finely powdered carmine in culture solution. The particles of carmine were wafted to the cytostome. The granules of carmine were collected at the base of the cytostome exactly as were the bacteria in the cases cited above, but the carmine grains were packed much more loosely together. Like the bacterial balls, the carmine balls moved from the base of the cytostome forward, but instead of passing right to the anterior end they passed inwards, as shown in Text-fig. 3, and then continued backwards down the opposite side of the body to

the mouth. They were evacuated in the same manner as were the remains of the nutritious particles. Smaller clumps of carmine move more rapidly than larger clumps.

In cases where the animals were full of balls of nutritious material before feeding, it could be seen that the carmine balls moved backwards more rapidly than did nutritious balls.

A similar experiment in which Indian ink was used instead of carmine gave identical results.

TEXT-FIGS. 3 AND 4.
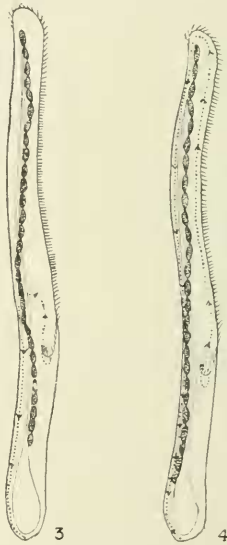
The above observations differ from those of Lund (**16**) on Bursaria, in that in Spirostomum the ingested material follows a definite course through the body. When the material ingested is of a nutritious nature, the balls travel to the anterior end and then move backwards following a course parallel to the meganucleus. In her work upon the food vacuoles of Car-chesium, Greenwood (**8**) states that the food vacuoles travel round the meganucleus. In Spirostomum ambiguum, and in Paramoecium also, according to Metalnikov (**20**), substances of no nutritive value take a much shorter course,

move much more rapidly, and are therefore expelled in a much shorter time than nutritious ones.

A suspension of hard-boiled yolk of egg in water was fed to some Spirostoma. The particles ingested were observed to follow the course taken by carmine granules and Indian ink. A similar course was followed by globules of raw milk. From this it seems apparent that raw milk and yolk-granules are not nutritious to Spirostomum ambiguum, the animal evidently being unable to digest them.

6. Varieties of Spirostomum ambiguum.

During the observations made upon a number of different cultures of Spirostomum ambiguum it soon became evident that two distinct varieties were present. That these were not stages in a developmental cycle was proved by making pure cultures of each.

Stein (**28**) describes a number of varieties of which his Pl. ii, fig. 10, and Pl. iii, fig. 3, show two chief types. These two main varieties, corresponding to the ones present in the cultures, are also recognized by Roux (**26**), and are termed by him Spirostomum ambiguum major and Spiro-stomum ambiguum minor. These two varieties differ from one another in a number of important details, the most striking of which is size.

The major variety is usually much broader in proportion to its length than the latter. The average length of the ordinary members of the major variety, when fixed with warm Schau-dinn's solution, is 800–900 μ, whilst that of ordinary members of the minor variety, when treated in the same way, is 400–500 μ. The posterior end of the minor variety is truncated, whilst that of the major variety is rounded. The protoplasm of the major variety is yellowish in colour, whilst that of the minor variety is greyish white and the endoplasm is less coarsely vacuolated and more granular in the latter than in the former. In the major variety the peristomial membranellae extend from the extreme anterior end to some point posterior to the middle of the animal's body. In some individuals of

the major variety the mouth may be situated almost at the extreme posterior end, about level with the middle of the contractile vacuole. This variation in the position of the mouth depends, as will be seen later, upon the degree of growth to which the animal has attained since the last fission. In the minor variety the peristomial membranellae extend from the extreme anterior end backwards to the mouth, which usually is in the anterior third of the body length.

The shape of the contractile vacuole also differs in each form. In Spirostomum ambiguum major it is a pear-shaped vessel, almost as broad as the animal, but never occupying more than an eighth of the animal's length. In the minor variety it is much larger, generally filling, when fully expanded, the posterior quarter or even third of the animal. In some cases it has been observed to fill half the total length. The shape of the contractile vacuole in the minor variety tends towards an oblong.

The time between contractions of the vacuoles in the two varieties varies. Thus, in the major variety the average time obtained from repeated observations on a large number of animals from four different cultures was every eight and a half minutes. The maximum period between contractions which was ever observed was ten minutes and the minimum seven minutes. In the minor variety the average was sixteen minutes. On two occasions thirty and thirty-four minutes respectively elapsed between contraction of the vacuoles of individuals of this variety. Since these two figures were so different from the rest they were not included in the average.

The meganucleus in both varieties is similar in form and structure, but I have never seen large, multi-vacuolated macrosomes in the meganucleus of the small variety. Since the contractile vacuole is very large, the length of the meganucleus in proportion to the body length in the small variety is less than in the large variety.

The micronuclei in the small variety are similar to those of the majority variety except that they are smaller and often rather disc-shaped.

The chief differences between the two varieties, therefore, can be summarized as difference in size, in colour of protoplasm, in position of mouth and length of peristomial area, and finally in the shape and relative size of the contractile vacuole and in its periods of contraction.

7. REPRODUCTION.

A. Observations on the Growth and Reproduction of Spirostomum ambiguum during Cultivation.

In many cultures of the large variety of Spirostomum ambiguum (see p. 404) made by both the culture methods described above, it was observed that the size of the individuals in different cultures varied enormously. Observations made upon animals grown in a rich wheat or leaf culture medium and kept at a constant temperature of 16° C. in the incubator, showed that they were larger than those grown in a similar culture at a higher temperature. Even when the culture was not very rich in food and was kept at a temperature of about 16° C. the individuals were very large. That these differences in size were not due to differences of race in the Spirostoma was proved by reversing the conditions, when the animals altered in size correspondingly.

Further, it was observed that individuals in the cultures which were kept at 20° C. divided more rapidly than did those in cultures kept at 16° C. or lower. This was confirmed by starting two cultures similar in every way and containing the same number of Spirostoma but keeping one at 20° C. and the other at 16° C. The former increased more rapidly than the latter.

This is probably the whole reason for the variations in size found in Spirostoma ambiguum major in the various cultures. When the cultural conditions favour rapidly repeated divisions the individuals become smaller than the normal size of the species. At each division their size is halved and the intervals between successive divisions are so short that they

are unable to reach normal size again before the next division occurs. After this division they are therefore still smaller than half the normal, and this decrease in size is progressive, producing a culture full of small individuals.

On the other hand, in other cultures the stimulus to division was evidently weak or in abeyance, although assimilation and growth were in no way impaired. The Spirostoma divided, therefore, infrequently, and grew to far beyond the average size, sometimes attaining as much as two and a half times the size of the ordinary individuals.

In starving cultures also, or in tap-water, they become very small. The cytoplasm seems to decrease more rapidly than the nucleus, since in such animals the nucleus is much coiled together.

A curious phenomenon, observed in most cultures on certain occasions, was the agglomeration of many Spirostoma into balls and strings. In an undisturbed condition of such a culture these clumps were suspended in the fluid, but they sank to the bottom if they were agitated. This condition was very marked at the time of conjugation in nearly all the cultures which contained conjugants. Lebedew (15) describes a similar massing together of individuals of Trachelocerca in the material from which he obtained his conjugants. He believed that the animals congregated around food-bodies. I have seldom been able to find any food forming the nucleus of the balls occurring in my cultures. Calkins (4) states that in Uroleptus mobilis epidemics of conjugation 'are invariably preceded by a characteristic massing or agglomeration of individuals'. By transferring these masses to other dishes containing fresh culture medium, he invariably obtained epidemics of conjugation.

In many cultures Spirostoma were observed adhering to the sides of the tubes, evidently by the mucous secretion described in this animal by Jennings (12). It is possible gently to draw them away a little from the solid to which they adhere without severing the mucus. I am of the opinion that the suspended agglomerations are formed in a similar way, by

the adhesion of many animals by a mucous secretion, and that this condition is particularly prevalent at times of conjugation. Possibly by its means the future conjugants first adhere one to another before any protoplasmic connexion is established.

Some attempts were made to induce conjugation in the large variety of Spirostomum by experimental means.

Maupas (19) produced conjugation in S. teres by subjecting them to alternations of a rich diet and a period of semi-starvation. A similar process was tried with Spirostomum ambiguum but without any result.

In his work upon the conditions for conjugation in Paramoecium, Hopkins (11) has produced conjugation by subjecting the animals to a preliminary period of semi-starvation for about two weeks and then adding food and a small percentage of various solutions of inorganic salts. The salt solutions used were :

0·00002 N solution of ferric chloride,
0·00025 N solution of potassium chloride,
0·001 N solution of sodium chloride,
0·0001–0·0004 N solution of calcium nitrate.

Somewhat similar experiments have been done by Zweibaum (32). He, too, has been able to produce conjugation, and has found that ferric chloride gives the best results. I was unable, however, to induce conjugation in Spirostomum by any of these methods. Ferric chloride, in the quantities used by Hopkins, was an excellent stimulant to division, and cultures treated in this way gave rise to great numbers of very small Spirostoma, which, some weeks later, presumably when the effects of the salt had been lost, returned gradually to a normal size.

Conjugation was first observed in the large variety of Spirostomum ambiguum on May 4, 1922. It occurred in a wheat culture which had been kept at a constant temperature of 20° C. since March 13. On May 7 conjugating pairs were observed in two other wheat cultures, both of which had also been kept at the above constant temperature. One culture dated from January 4 and the other from the end of

March.   All three cultures were quite normal, no experi-
ments having been performed upon them to induce con-
jugation. There was no appearance of a true epidemic such as
Mulsow (**21**) found in Stentor coeruleus and Stentor
polymorphus in May 1911, when he obtained some 2,000–
3,000 pairs of conjugants. A few pairs were observed each
morning in each culture for about a fortnight, and after that
the cultures again became normal.

For about a fortnight previous to conjugation being observed,
microscopic observation of a few individuals taken at random
from these cultures had shown that the protoplasm had
become dark in colour and somewhat granular in appearance.
But the protoplasm of all conjugants observed was of the normal
light colour. Whether the protoplasm darkens in colour
previous to conjugation and then grows light again when this
takes place, or whether individuals destined to conjugate
remain light, in which case we must suppose that all such
individuals had escaped my observation, I cannot say with
certainty. From the fact that in some leaf cultures (see below)
the protoplasm of all individuals observed was dark, and that
later almost all individuals in the culture conjugated, the former
suggestion seems to be the more probable.

Since all these cultures had been kept at a constant tempera-
ture, it was impossible that the sudden rise in room tempera-
tures, which took place about the above dates, could have
stimulated the Spirostoma to conjugate.

On May 24 a number of conjugating individuals were
observed in one wheat culture and in two leaf cultures which
had been kept always at the ordinary laboratory temperature,
then about 22° C. The period of conjugation lasted for about
ten days. In two of the cultures only a few pairs were found
to be conjugating, but in one leaf culture on an average five
or six pairs were removed each morning. This gave a fairly high
percentage of conjugating individuals. That the cultures were
in a good condition was shown by the fact that division took
place frequently in the non-conjugating organisms.

In his paper upon the conjugation of the Stentors Mulsow (**21**)

concludes that it was unfavourable conditions in their environment which caused them to conjugate, since, when the conjugants were removed, all non-conjugants left in the cultures died within a few days. That this did not apply to my cultures of Spirostoma was shown by the fact that the non-conjugants continued to live quite normally when left in the undisturbed cultures after the period of conjugation had passed. The fact that conjugation took place in both wheat and leaf cultures was interesting, since it indicated that the raw material from which the culture medium was made was not the factor inducing conjugation. It is probable, however, that the physical and chemical constitution of an extract of leaves is not so widely different from that of an extract of wheat that it would affect the behaviour of the organisms in this respect. This point also seems worthy of more detailed investigation, since Baitsell (1) found that in pedigreed cultures of Stylonichia pustulata conjugation occurred on two occasions in animals kept in a beef medium, whereas it never occurred in those forms kept in hay infusion though they were identical in age to the former. From this he concludes that conjugation is induced by external conditions affecting the organism and that it bears no relation, in this form at least, to a particular period of a ' life-cycle '.

Since all the cultures in which conjugation had so far taken place had been stocked from the descendants of Spirostoma obtained on one occasion from a pond near Styal (near Manchester) in July 1921, it was at first thought possible that the length of time during which the animals had been cultivated might be a factor influencing conjugation. It is a well-known theory that Protozoa multiply for a long period without conjugation, after which the rate of multiplication decreases and a period of depression ensues in which the animals degenerate and die unless they are stimulated to renewed division by conjugation. This theory was first suggested by Maupas (19).

From June 14 to June 22, however, conjugation was observed in a leaf culture of Spirostomum ambiguum which had

been collected from a pond at Hale in Cheshire only a fortnight before. This new culture had been kept in the incubator and had divided repeatedly. Moreover, the individuals which were not conjugating were dividing actively during the period of conjugation. The proportion of conjugants was greater in this culture than in any other. The culture was started from about ten individuals, and, since approximately forty pairs of conjugants were removed during the third week, the conjugants must have been capable of repeated division immediately prior to conjugation, in which case a senile condition was impossible. That conjugation took place in a culture where the division rate was high was found by Baitsell (**1**) in Stylonichia pustulata, but, whereas this culture of Spirostomum ambiguum continued to flourish after the period of conjugation had passed, the non-conjugants in the culture of Stylonichia pustulata became degenerate and died out.

In one of two of the wheat cultures in which conjugation was observed, numerous Colpidia and Paramoecia were present in addition to Spirostomum ambiguum. Conjugation was never observed among any members of the two former species. Whatever the conditions might be inducing conjugation in Spirostomum ambiguum, they did not have the same effect on the Paramoecia and Colpidia.

My experiments and observations have not, therefore, up to the present time, thrown any new light upon the factors causing conjugation. They seem to indicate that the seasonal factor is an important one, in Spirostomum ambiguum at any rate, and I must hope that further and more detailed work will enable me to follow out such hints as I have so far gained. An important part of such work would be the study of this organism in its natural surroundings in its native ponds and ditches.

B. Fission.

It is to Stein (**28**) that we are indebted for the first description and figures of fission in Spirostomum ambiguum. He observed the phenomenon in four cases, three of which were

in the small variety and one in the large variety. As he relied upon freshly collected material this was not strange, for even in healthy cultures individuals undergoing division often are not numerous. This is, no doubt, due to the fact that a period of two to three days elapses between divisions even in an animal subjected, as far as it is possible for us to tell, to excellent conditions. Divisions takes place during the night as well as in the day time ; for in the morning usually there are present in the cultures a number of animals in the last stages of division, or some which have recently divided.

 As the process of division differs very little in the two varieties, it is unnecessary to give a description of each ; it will be sufficient to describe it as it occurs in the major variety and to note any deviation which occurs in the minor variety.

In order to follow the entire process of division it was found best to isolate individuals which showed the first signs of coming fission and to observe them through the whole process. Stained preparations were made at different stages.

The time taken to complete the phenomenon varies in different individuals, but the average time is from seven to eight and a half hours : even then, the two daughters, though separated, have not attained the normal form. This is especially true of the form of the meganucleus. Simpson (27) gives one to two hours as the time required for division in Spirostomum ambiguum; but this I take to mean the actual division of the animal's body into two parts irrespective of nuclear changes.

In the major variety the mouth, in animals about to undergo division, lies midway between the anterior and posterior ends. Large numbers of animals belonging to the major variety have been observed undergoing division, and in every case the mouth of the parent was at the middle of the body length. In his single observation on fission in this variety Stein describes the old peristome as extending through the anterior two-thirds of the body length, and the new peristome of the future daughter as developing in the posterior third of the body. He further states that after half an hour's observation the beginning of

the division of the body showed between the old and new peristomes. In such cases division would be unequal; one daughter would be two-thirds the length of the other. On no occasion have I seen any such inequality in size between the two daughters.

In the individuals of the small variety about to undergo fission the old peristome lies in the anterior third and the new peristome forms in the middle to posterior third of the body.

Animals about to divide are always much longer than the average-sized individual of the same culture. This length is reached by a gradual process of growth, principally in the region behind the mouth.

The first indication of coming fission is cytoplasmic, not nuclear. It consists of the formation of the peristomial membranellae of the new cytostome. In the major variety the anterior end of the new membranellae is almost level with the posterior curve of the old. The first indication of the formation of these daughter membranellae is a slight ridge in the posterior half of the body, running parallel with the rows of the body cilia. This ridge gradually becomes more pronounced, and along it the new membranellae are formed. At first these are very small but rapidly grow larger. The immature membranellae are much shorter in proportion to their width than are the mature ones. This gives them a somewhat leaf-like appearance. The movements of these developing membranellae, almost until the separation of the two daughters, are very irregular, many of the individual membranellae moving in different directions to their neighbours, which gives the whole a ragged appearance. This lack of co-ordination in movement is very noticeable when compared with the steady, undulating motion of the mature membranellae.

The formation of the membranellae is usually well advanced before the nucleus shows any intimation of approaching fission. The times given above were taken from the beginning of the nuclear changes. The somewhat zigzag form of the meganucleus becomes straightened, the lobation is gradually lost, and its shape becomes vermiform. Stained preparations of this stage

show that the granular structure of the nucleus is quite normal. The micronuclei, too, are normal (Text-fig. 5).

The meganucleus next begins to contract and incidentally to thicken. In some individuals contraction begins before the lobation is completely lost, but in the majority of cases the former is the normal method. In almost all individuals the anterior part of the meganucleus remains in its normal

TEXT-FIGS. 5, 6, 7, 8, AND 9.
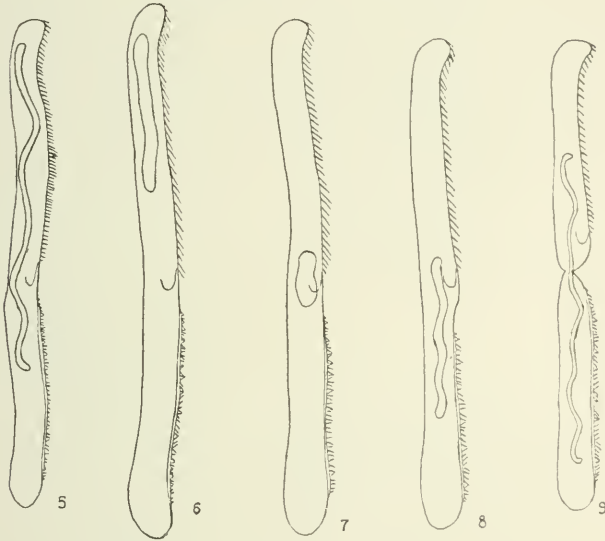
position, and the posterior and middle parts contract towards it in such a way that the almost completely contracted meganucleus lies in the anterior end of the body (Text-fig. 6). Its shape is still vermiform but much thickened and less than a quarter of the length of the body.

All the sections which have been studied of animals at this stage show darkly stained granules irregularly dispersed over a mesh-work. These meshes do not combine with stains so strongly as do the granules. Preparations of stages intermediate between the expanded and the anteriorly contracted stages of the meganucleus show that the micronuclei move

forwards with the meganucleus, but that they become much
swollen and stain very lightly (Pl. 22, fig. 4, *M.N.*). At this
stage, when the meganucleus lies in the anterior end, the micro-
nuclei are almost three times their normal size. They are
surrounded by a distinct halo. Some are close to the mega-
nucleus, but others are a slight distance away from it.

The meganucleus moves backwards until it comes to lie in
the middle of the animal's length, at the same time contracting
still further (Text-fig. 7). The movement backwards to the
middle of the body is fairly rapid. Here the meganucleus
remains for a relatively much longer period. In appearance
it is a roughly oval, dense structure. Its form is not rigid
but slowly changes, showing protuberances at the side which
gradually disappear and reappear at other places.

In many sections of animals with the meganucleus fully
contracted in the centre of the body it has not been possible
to find any micronuclei. In one or two specimens large,
pale-staining micronuclei have been seen ; these evidently
have not yet undergone division. In other cases small, intensely
staining micronuclei have been found near the meganucleus.
It seems quite probable that these are the products of division,
and I hope to say more about them in a further paper. In all
further stages of division in Spirostomum ambiguum
the micronuclei are small and stain normally.

From the fact that, when the micronuclei of conjugants
are about to divide, they become swollen and similar in appear-
ance to the ones described above, it is to be concluded that
these micronuclei divide some time during the migration back-
wards of the anteriorly contracted meganucleus, or soon after
it takes up its central position. Unfortunately I have never
actually seen any division taking place in any preparations,
and conclude therefore that the division must take place very
rapidly. A similar difficulty was experienced by Johnson (13)
whilst working upon the division of Stentor.

During the time when the meganucleus is fully contracted
in the centre of the body, a slight dilation becomes visible
in the feeding canal of the contractile vacuole, on a level

with the anterior end of the meganucleus. This is the beginning of the daughter contractile vacuole.

A gradual elongation of the meganucleus next takes place (Text-fig. 8). A curious phenomenon in the elongation of the meganucleus, observable also in its contraction, is that this process does not take place equally fast at each end of the mass. Elongation takes place more rapidly in the posterior part than in the anterior, so that while the posterior half of the animal has quite a long developing meganucleus only a short part projects into the anterior half. The elongating meganucleus does not expand in a straight line but is often coiled in its course. Both anterior and posterior ends have the shape of a crook, which often persists even after the separation of the two daughters.

During the elongation of the meganucleus a slight constriction of the body can be observed a little posterior to the anterior cytostome. This gradually becomes more pronounced and marks the point of the future separation of the two daughters.

Coincident with the development of this constriction is the gradual enlargement of the dilation in the feeding canal of the contractile vacuole. For some time it continues to empty with the contents of the original vacuole, but a considerable length of time before the separation of the two daughters it becomes disconnected from the posterior part of the canal and contracts independently of the posterior vacuole. This separation of the anterior contractile vacuole from the posterior one seems to take place as soon as the constriction of the cytoplasm is sufficiently deep to allow the excretion of the fluid through a pore in the median line of the constriction.

Although the meganucleus in the anterior half seems to grow more rapidly immediately prior to the separation of the two daughters than that in the posterior part, the length of meganucleus in each daughter at the time of separation is still a trifle unequal (Text-fig. 9). This inequality seems to be adjusted later. The meganucleus divides into the two

daughter meganuclei just before the cytological separation of the two daughters takes place.

The newly separated daughters can be distinguished from ordinary individuals by the fact that they are shorter ; and also the meganucleus is vermiform and not moniliform. Further, the cytosome is always at the posterior end, and the peristomial membranellae therefore extend practically the whole of the animal's length. Since growth takes place much more rapidly behind the cytostome than in front of it, the cytostome appears to move forwards gradually, so that in animals about to undergo fission it is central in position.

Lobation of the meganucleus seems to take place at varying times after the separation of the daughters. There seems to be no correlation at all between the number of micronuclei present and the number of lobes formed in the meganucleus. It seems probable, as Collin suggested for the nuclei of Acinetaria, that lobation is governed by the varying tensions of the nuclear membrane.

C. Conjugation in the Major Variety of Spirostomum ambiguum.

Stein (**28**) observed conjugation in Spirostomum ambiguum on July 28, 1857. Balbiani also observed it in this species. Conjugation, as stated in Part 7, was seen by the present worker in a number of individuals of the major variety of this species during May and June 1922.

It was noticed that the conjugants were considerably smaller than the ordinary individuals. Stein describes a similar condition in his specimens. It was also noticed that the pairs of conjugants in any one culture showed less variation in size than non-conjugants of the same culture. The greater amount of variation in size in the non-conjugants was probably due largely to the fact that all stages in growth between newly separated daughters and individuals about to divide were present, whereas, from the fact that the cytostome was always central in position with regard to the body-length, it was

apparent that the conjugants were all at the final stage of growth when division should commence. No information throwing light on the means by which the smallness in size in the conjugants is arrived at, was obtained. In his biometrical study of conjugation in Paramoecium caudatum Dr. Raymond Pearl (22) found ' that conjugant individuals when compared with non-conjugants were shorter and narrower and less variable both in length and breadth '. He also showed that there was a high degree of correlation between the lengths of the two members of conjugant pairs. He proved, by numerous careful measurements, that such a high degree of homogamic correlation was not due to the random pairing of individuals in a ' homogeneous population of low variability '. Miss Watters (30) has obtained similar results with regard to the relationship in size between conjugants and non-conjugants in Blepharisma undulans. Such rough observations as have been made during the present study of conjugation seem to indicate that similar relationships exist between the conjugants themselves and between the conjugants and the non-conjugants in cultures of Spirostomum ambiguum.

Both conjugants are, as was noticed by Stein (28), attached along the peristomial groove. This makes the taking in of food during conjugation impossible. From the fact that conjugants, even in the earliest stages of conjugation, are rarely found containing ingested food, it would appear that ingestion ceases some time prior to conjugation.

The peristomial membranellae are not absorbed during conjugation. Generally the anterior end of one individual of the pair is attached to a point a little posterior to the anterior end of the peristomial groove of the other. The attachment ends at the cytostome. Since the cytostome is central in position, it follows that conjugating individuals are attached for half the body-length. Stein (28) in his figure of a pair of conjugants depicts them as being attached to one another from the extreme anterior end of each. Quite a number of pairs attached in this way have been met with during the present observations, but the method of attachment in the majority

has been in the manner described above. When they are attached in the manner described by Stein (**28**) the posterior ends are level, since the conjugants are almost without exception equal in size; when they are attached in the more common manner, however, the posterior end of one individual projects beyond that of the other. Observations of individuals just beginning to conjugate showed that the anterior ends were the first to become attached. Sections show that the conjugants are joined by a thin sheet of ectoplasm and that the endoplasm of the individuals does not mingle.

The contractile vacuole seems to function in a normal manner during conjugation, though the average time of its contraction was not studied.

One of the difficulties encountered in studying conjugating pairs is that newly attached individuals often become separated in drawing them up a pipette. Such severed conjugants have never been observed to become reattached but sooner or later die. Maupas (**19**) experienced similar difficulties whilst working upon conjugation in S. teres. Whilst working upon Paramoecium, however, Calkins found that conjugants, if severed before there had been any exchange of nuclear material, would live and divide in a normal manner. Similar experiments upon artificially severed conjugants were carried out by Baitsell (**1**), but without exception the severed conjugants all degenerated and died within the twenty-four hours following the operation.

The time taken from the attachment of a pair of conjugants to the time of their separation varies between sixty and seventy-two hours. It is very difficult to be absolutely certain of the duration of conjugation in a pair, since it is practically impossible to remove for observation a pair which are only just becoming attached. They invariably become separated during the removal from the culture to the depression slide.

In order to secure permanent preparations of as many stages of conjugation as possible, the conjugants were removed from the culture and placed into test-tubes, which contained some of the culture solution, and fixed at different intervals. This

unfortunately entailed a rather high death-rate, but it seemed unavoidable. Further, the supply of material was meagre and the technical difficulties considerable.

For an hour or two subsequent to the attachment there was no change in the meganuclei or micronuclei of either conjugant.

The first big change to take place was the breaking up of the meganucleus into isolated segments by the snapping of its commissures (Pls. 22 and 23, figs. 7 and 13). The greater number of the segments of the meganucleus migrated towards the anterior end of the conjugant's body and came to lie in the area opposite to the line of attachment. A few of the posterior segments invariably remained in the neighbourhood of the contractile vacuole and never migrated forwards. The fact that the meganucleus became fragmented during conjugation was noticed and figured by Stein (28). He did not describe it as actually fragmenting, the pair of conjugants upon which he worked evidently having passed this stage when he first observed them.

In some preparations made before fragmentation of the meganucleus and in almost all the ones made afterwards, vacuoles were observed in the substance of the meganucleus. These vacuoles varied in size, sometimes attaining to half the size of the lobes of the meganucleus. Sometimes only one vacuole was present in each lobe, but in other cases three or four were present. The vacuoles often projected, causing the nuclear membrane to bulge outwards. In stained preparations these vacuoles were colourless, but lightly staining spheres could be seen in the centre of some of them (Pl. 22, fig. 5, *vac.* and *p.*). I am unable to offer any explanation of the nature of these spheres. They were present at all stages from the fragmentation of the meganucleus to the early stage of the exconjugant. They were evidently a product of the degeneration taking place in the meganucleus, and might result from the coagulation by the fixative of some fluid in the vacuole. This suggestion unfortunately is not very plausible, since it demands either that the spheres should be present in all the vacuoles, which was not the case, or else that the contents

of different vacuoles varies, which seems a very improbable hypothesis.

These vacuoles, present in the meganucleus during conjugation, are not to be associated in any way with the vacuoles inside the larger ' macrosomes ' of a normal meganucleus. The former were found in the interstices of the granular structure and represent a vacuolation of the nuclear sap ; whereas the latter appeared within the substance of the ' macrosomes '. Although small macrosomes were always present in the meganucleus of conjugants, no large nor vacuolated ones were ever seen in the degenerating meganuclei of conjugants. It is almost unnecessary to remark that this does not mean that the presence of large vacuolated macrosomes can never be coincident with conjugation, but merely that, in the comparatively small number of conjugants that have been studied, they have not been present. However, if the vacuolated-macrosome condition is to be regarded as being caused by an unknown factor in the culture, it may be that this same factor is not suitable for inducing conjugation.

No further visible changes took place in the meganucleus until after the separation of the conjugants.

In newly separated exconjugants the fragments of meganucleus were present and stained as intensely as in conjugants. These fragments contained vacuoles, as did those in the conjugants. In preparations made at a slightly later stage the vacuoles had become more pronounced and now bulged outwards greatly. In some specimens they appeared to have burst, for circular cavities could be seen in the fragments of meganucleus.

Absorption of the old meganucleus took place during the first few days subsequent to separation. Gradually the fragments took up the stain less intensely, and often a clear space could be seen in the cytoplasm surrounding them. Not all the fragments were absorbed at the same time. Complete absorption of the meganucleus did not take place until the rudiments of the new meganucleus had attained to a considerable size (Pl. 22, fig. 6, $L.$ and $A. A.$).

The first stages in the formation of the rudiments of the meganucleus have not been seen. Preparation of the earliest stages obtained showed two or more thin discs about the middle of the exconjugant. Since these discs were denser than the surrounding cytoplasm, they could be seen in living exconjugants. The ground substance of these disc-shaped rudiments of the meganucleus stained very feebly, scarcely more intensely than did the surrounding cytoplasm. They contained a number of deeply staining granules. In the earlier stages these granules were distributed through the interior of the disc, but later they appeared to migrate to its periphery. Some of these granules showed one or more vacuoles inside them and seemed to be identical to the macrocomes of the meganucleus of normal individuals of Spirostomum ambiguum (Pl. 22, fig. 8, M.V.).

The normal number of these meganuclear discs present in the exconjugants was two. Occasionally four, and in one preparation six, were seen. Whether these large numbers arose by division of an original two, or whether the normal two were formed first, and later nuclei, which normally remain as micronuclei, became converted into them, is not clear.

Although I succeeded in keeping exconjugants alive in test-tubes until seventeen days after the separation of the conjugants, there were no signs of constrictions appearing in the rudiments of the meganucleus to form the moniliform meganucleus. The few individuals that remained alive so long died after seventeen days, and the cessation of conjugation left me without any material with which to make another attempt.

Owing to the small quantity of material at my disposal, the smallness in size of the micronuclei, their great number, and the difficulties of staining them whilst undergoing division, my observations upon them are very fragmentary, a fault which I hope to rectify in an additional paper.

The first change in the micronuclei during conjugation occurred some time after the fusion of the conjugants and before the severing of the commissures of the meganuclei. This change consisted of the gradual swelling up of the majority

of the micronuclei whilst still in their old position close to the meganucleus. Their staining powers decreased as their size increased.

The increase in bulk of the chromatin sphere of the micronucleus did not take place at the expense of the surrounding halo, since this too increased in size and appeared as a wide, clear area surrounding the swollen micronuclei (Pl. 23, fig. 13). It does not seem probable, therefore, that the increase in size of the micronuclei was due to the absorption of fluid from the surrounding halo, unless the latter obtains fresh liquid from the surrounding cytoplasm. As they increase in size the micronuclei move a little distance away from the meganuclei.

In a large number of the animals studied a few of the micronuclei, particularly those situated towards the posterior end of the conjugant, appeared to be unaffected by this change, and they retained their minute size. Such micronuclei were often near to isolated fragments of meganucleus at the posterior end, even after the majority of the micronuclei were well advanced in division. Their fate was not known, but since they were never present in the exconjugant immediately after separation, one may presume that they were subsequently absorbed.

When the majority of the isolated segments of the meganucleus migrated towards the anterior end of the conjugants (see p. 425), the greater number of the swollen micronuclei performed a similar migration and came to lie in the cytoplasm between the scattered lobes of the meganucleus. A few of the swollen micronuclei remained amongst the lobes of the meganucleus at the posterior end of the body. These underwent the same changes as did those at the anterior end.

In their new position the micronuclei continued to swell. Since the amount of chromatin did not increase, but was merely distributed through a greater bulk, the micronuclei became very pale and difficult to study. When they had attained to their greatest size the swollen spheres began to stain unevenly as though the chromatin was becoming aggregated at certain points (Pl. 23, fig. 12). It then became apparent

that the chromatin was gathering at one side of the halo (Pl. 23, fig. 16), the rest of the micronucleus being almost colourless. At the opposite side of the micronucleus to the aggregation of chromatin a projection appeared, lengthening until it touched the membrane at the edge of the halo. Threads formed between the chromatin aggregation, which now became broken up into definite granules, and the apex of the projection (Pl. 23, fig. 11). This I took to be the beginning of the formation of a spindle ; but, in his work upon Stentor, Mulsow (21) states that such a condition may be preparatory to the formation of the spindle or a stage in degeneration prior to absorption of the micronuclei, both stages being remarkably alike.

Thus it is not possible to state with certainty that all micronuclei in this stage gave rise subsequently to spindles. No stages in the formation of the second pole were discovered.

The formation of the spindle took place inside the micronuclear membrane. The structure took up the entire space and no halo was to be seen. Many of the spindles were almost globular in shape (Pl. 23, figs. 10 and 15) ; a few, however, were more diamond-shaped (Pl. 23, fig. 9). In the globular form the apices were flattened, but in the others they were quite pointed. Whether this difference in shape was accidental or was the expression of different stages in the division process is not certain, but the latter interpretation seems to me more probable.

No centrioles were ever seen. The spindle-fibres were usually quite obvious. They appeared to fuse and form groups when approaching the apex of the spindle. In the diamond-shaped spindle the fibres appeared to converge at one point, but in the globular spindles the groups of fibres did not all come together at the pole (Pl. 23, figs. 10 and 15).

Spindles were present at the same time in both conjugants, but division did not take place simultaneously in all the micronuclei of the conjugant, since, besides spindles at the equatorial plate stage, micronuclei at the swollen stage prior to division (see Pl. 23, fig. 12) were present, as were also micronuclei which were not so swollen or pale. Some of these

very swollen micronuclei possibly were about to degenerate and not to divide. No stages demonstrating the formation and crossing over of the gamete nuclei nor the degeneration of the surplus ones have been obtained.

In a preparation of an exconjugant, made soon after its separation, a spindle was seen at the anterior end of the body. This spindle (see Pl. 23, fig. 14) was more elongated than those present in the conjugants. It was immediately surrounded by denser protoplasm than that of which the rest of the body was composed. It also was at the equatorial plate stage. A very careful investigation of the rest of the animal did not reveal the presence of other spindles nor of other micronuclei. This spindle was therefore regarded as that of the zygote nucleus, and it was concluded that the other micronuclei had all degenerated. Subsequent stages in division of the nuclei in the exconjugant were not seen.

No further stages of the division of the micronuclei were discovered in the exconjugants. In two exconjugants four, and in one preparation eight, micronuclei were discovered in the neighbourhood of the two rudiments of the meganucleus. In no preparations were micronuclei found attached to the edge of the meganuclear discs as Mulsow found in exconjugants of Stentor.

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9. EXPLANATION OF PLATES 22 AND 23.

Fig. 1.—Part of the meganucleus of an ordinary individual. *L.*, a lobe of the meganucleus. *C.*, a commissure. *M.N.*, a micronucleus surrounded by a halo. *m.*, a non-vacuolated macrosome. *vac.*, a macrosome containing a small vacuole. × 1200.

Fig. 2.—Part of the meganucleus of a normal individual. *MV.*, large macrosome. *vac.*, vacuole inside the macrosome. × 1200.

Fig. 3.—The terminal lobe of a similar meganucleus. × 1200.

Fig. 4.—Part of the meganucleus of an ordinary individual undergoing fission, with the meganucleus contracted towards the anterior end of the body. *X.*, the anterior end of the meganucleus. *M.N.*, swollen micronuclei surrounded by halos. × 1200.

Fig. 5.—Isolated lobes of the fragmented meganucleus of a conjugant. *vac.*, vacuoles inside the lobes. *p.*, pale-staining spheres appearing inside some of these vacuoles. × 1200.

Fig. 6.—The nuclear apparatus of a young exconjugant. *L.*, the darkly staining fragments of the old meganucleus. *A.*, the newly forming meganuclei, as yet very pale. The presence of as many as five newly forming meganuclei in one exconjugant is unusual. × 1200.

Fig. 7.—A preparation of a pair of conjugants showing the meganucleus in a fragmented condition. × 120.

Fig. 8.—One single large sphere of the newly forming meganucleus present in an exconjugant. *MV.*, macrosome-like bodies. × 1200.

Fig. 9.—Pointed spindle of a micronucleus in a conjugant. × 1200.

Fig. 10.—Globular spindle of a micronucleus in a conjugant. × 1200.

Fig. 11.—Swollen micronucleus with unevenly distributed chromatin at one side and spindle-threads forming at the other. × 1200.

Fig. 12.—Part of a conjugant. *M.N.*, micronuclei very much swollen. The distribution of chromatin in the swollen micronuclei is very uneven. *M.x.*, a micronucleus which is less swollen. *L.*, isolated lobes of the old meganucleus. × 1200.

Fig. 13.—Part of a conjugant. *L.*, isolated lobes of the meganucleus. *M.N.*, micronuclei which have begun to increase in size but are still close to the lobes of the meganucleus.   ×1200.

Fig. 14.—The spindle of the dividing zygote nucleus in an exconjugant. ×1200.

Fig. 15. *A.* and *B.*, division spindles of micronuclei in a conjugant. Spindle *A.* has flattened apices.   ×1200.

Fig. 16.—*M.S.*, a micronucleus approaching the spindle stage.